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Citation for published version:

Marriott, HM, Jackson, LE, Wilkinson, TS, Simpson, AJ, Mitchell, TJ, Buttle, DJ, Cross, SS, Ince, PG, Hellewell, PG, Whyte, M & Dockrell, D 2008, 'Reactive oxygen species regulate neutrophil recruitment and survival in pneumococcal pneumonia', *American Journal of Respiratory and Critical Care Medicine*, vol. 177, no. 8, pp. 887-895. <https://doi.org/10.1164/rccm.200707-990OC>

Digital Object Identifier (DOI):

[10.1164/rccm.200707-990OC](https://doi.org/10.1164/rccm.200707-990OC)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

American Journal of Respiratory and Critical Care Medicine

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Reactive Oxygen Species Regulate Neutrophil Recruitment and Survival in Pneumococcal Pneumonia

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Rationale: The role of NADPH oxidase activation in pneumonia is complex because reactive oxygen species contribute to both microbial killing and regulation of the acute pulmonary infiltrate. The relative importance of each role remains poorly defined in community-acquired pneumonia.

Objectives: We evaluated the contribution of NADPH oxidase-derived reactive oxygen species to the pathogenesis of pneumococcal pneumonia, addressing both the contribution to microbial killing and regulation of the inflammatory response.

Methods: Mice deficient in the gp91^{phox} component of the phagocyte NADPH oxidase were studied after pneumococcal challenge.

Measurements and Main Results: gp91^{phox}−/− mice demonstrated no defect in microbial clearance as compared with wild-type C57BL/6 mice. A significant increase in bacterial clearance from the lungs of gp91^{phox}−/− mice was associated with increased numbers of neutrophils in the lung, lower rates of neutrophil apoptosis, and enhanced activation. Marked alterations in pulmonary cytokine/chemokine expression were also noted in the lungs of gp91^{phox}−/− mice, characterized by elevated levels of tumor necrosis factor-α, KC, macrophage inflammatory protein-2, monocyte chemoattractant protein-1, and IL-6. The greater numbers of neutrophils in gp91^{phox}−/− mice were not associated with increased lung injury. Levels of neutrophil elastase in bronchoalveolar lavage were not decreased in gp91^{phox}−/− mice.

Conclusions: During pneumococcal pneumonia, NADPH oxidase-derived reactive oxygen species are redundant for host defense but limit neutrophil recruitment and survival. Decreased NADPH oxidase-dependent reactive oxygen species production is well tolerated and improves disease outcome during pneumococcal pneumonia by removing neutrophils from the tight constraints of reactive oxygen species-mediated regulation.

Keywords: macrophages; apoptosis; *Streptococcus pneumoniae*; mice; reactive oxygen species

Individuals with chronic lung disease are susceptible to severe forms of community-acquired pneumonia and have increased mortality (1). The commonest cause of community-acquired pneumonia in this population is infection with *Streptococcus pneumoniae*, the pneumococcus (2). The successful resolution of bacterial pneumonia is dependent on a coordinated immune

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

NADPH oxidase activity is believed to be essential to microbial host defense for a wide range of pulmonary pathogens, although excessive production of reactive oxygen species may contribute to lung injury.

What This Study Adds to the Field

For the commonest cause of community-acquired pneumonia, pneumococcal pneumonia, NADPH oxidase activation is not required for antimicrobial host defense, and in its absence disease outcomes are improved.

response with neutrophil recruitment when resident defenses are overwhelmed (3). After clearance of bacteria, a resolution phase, mediated by apoptosis of recruited cells and clearance by phagocytosis, prevents tissue injury by activated neutrophils (4). Neutrophil persistence leads to lung injury, and nonresolution of a neutrophilic infiltrate in pneumonia can result in the acute respiratory distress syndrome (5). Neutrophils are both effectors of host defense and central arbiters of lung injury (6).

Neutrophils kill microorganisms, using both reactive oxygen species (ROS) and granule-associated proteases (7, 8). ROS production in neutrophils is largely dependent on the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase system (9). ROS were believed to be the major mechanism of neutrophil-mediated antimicrobial host defense (10). An alternative view proposes that ion fluxes, associated with activation of the NADPH oxidase system, activate granule-associated proteases to mediate bacterial killing (7). Neutrophil-derived ROS and granule-associated proteases both contribute to acute lung injury (11, 12).

In fatal cases of pneumococcal pneumonia there are often no viable bacteria, and a persistent neutrophilic infiltrate in the lung is frequently the only finding (13). A dysregulated inflammatory response and excessive production of microbicidal factors contribute to mortality. The traditional paradigm suggests that neutrophils producing ROS are important to bacterial clearance but excessive neutrophil recruitment and delayed apoptosis are harmful. Because there are many individual molecules that contribute to both microbial killing and lung injury, understanding the necessary and sufficient elements for each process, and the identification of molecules that make a disproportionate contribution to one or other process, in specific infections, are desirable in order to guide selective therapeutic manipulation of the inflammatory response in pneumonia.

Mice lacking the gp91^{phox} subunit of the NADPH oxidase have informed our understanding of the pathogenesis of pulmonary infections (14). Because neutrophil-derived ROS have

(Received in original form July 6, 2007; accepted in final form January 17, 2008)

Supported by a Wellcome Trust Senior Clinical Fellowship to D.H.D., no. 076945, a British Lung Foundation Fellowship to H.M.M., F05/7, and by the Sheffield Hospitals Charitable Trust research grant no. 7843.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177, pp 887–895, 2008

Originally Published in Press as DOI: 10.1164/rccm.200707-990OC on January 17, 2008
Internet address: www.atsjournals.org

important roles in antimicrobial host defense, regulation of the inflammatory response, and the induction of lung injury, we have studied pneumococcal pneumonia in these mice. We demonstrate that NADPH oxidase-dependent ROS are redundant for pneumococcal killing and in their absence granule-associated proteases are still released. In contrast, NADPH oxidase-dependent ROS regulate neutrophil recruitment, activation, and survival and have a negative impact on outcome. These results suggest a shifting paradigm for the role of ROS in pneumococcal pneumonia. Some of the results of these studies have been previously reported in the form of an abstract (15).

METHODS

Animals

gp91^{phox}^{-/-} mice on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) were maintained as a homozygous colony. C57BL/6 mice (Harlan, Indianapolis, IN) were used as wild-type control mice. Female mice aged 8–12 weeks were used. All animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 with local ethics approval.

Materials

Annexin-V-phycoerythrin (PE), Ly-6G-fluorescein isothiocyanate (FITC) (clone 1A8), CD18-PE, CD11b-PE, CD62L-PE, and isotype controls were from BD Pharmingen (Oxford, UK). TO-PRO-3 was from Molecular Probes (Leiden, The Netherlands).

Pneumococcal Infection Model

Pulmonary infection of mice was by direct tracheal instillation (3) with 10⁷ colony-forming units (cfu) of type 2 pneumococci, strain D39, and in specific experiments with type 2 pneumococci lacking pyruvate oxidase activity (SpxB⁻) (16) or type 1 pneumococci (strain SSISP; Statens Serum Institut, Copenhagen, Denmark), or mock infection with phosphate-buffered saline (PBS). Systemic infection was by intraperitoneal injection with 10⁴ cfu of D39 in 100 μ l of PBS.

Collection of Bronchoalveolar Lavage, Blood, and Lungs

Mice were killed by administration of an overdose of sodium pentobarbitone and exsanguination. Bronchoalveolar lavage (BAL), cell differentials using thin-layer cell preparations (Shandon Cytospin; Thermo Fisher Scientific, Waltham, MA), and viable bacterial counts in lung and blood were performed as described previously (3).

Detection of Apoptosis

Apoptosis detection was by annexin-V and TO-PRO-3 staining and flow cytometry, as a measure of phosphatidylserine translocation and cell viability, respectively, or by nuclear morphology on Cytospin preparations, as previously described (3). Neutrophils were identified by forward versus side scatter characteristics and Ly6G staining (3).

Neutrophil Activation

BAL neutrophil activation was determined by up-regulation of surface CD11b and CD18 and shedding of CD62L (*see the online supplement for details*).

Cytokine Production

Cytokines in BAL were measured with a mouse tumor necrosis factor (TNF)- α ELISA Ready-SET-Go! reagent set (eBioscience, San Diego, CA) or DuoSet ELISA development kits for mouse IL-6 and macrophage inflammatory protein (MIP)-2 (R&D Systems, Abingdon, UK), in accordance with the manufacturers' protocols. Limits of detection were 15 pg/ml. Alternatively, cytokines were measured by BD cytometric bead array (CBA) flex sets and BD FACSArray bioanalyzer (BD Biosciences, San Jose, CA), in accordance with the manufacturer's protocols. The limit of detection for KC, IL-12 p70, and IL-6 was 20 pg/ml and for MCP-1 and TNF- α it was 40 pg/ml.

Lung Injury

IgM and albumin levels in BAL were determined by ELISA (Bethyl Laboratories, Montgomery, TX) in accordance with the manufacturer's protocol.

Neutrophil Elastase Activity

Neutrophil elastase activity was measured in BAL (*see the online supplement for details*).

Myeloperoxidase Assay

Myeloperoxidase activity was assayed in lung homogenates (*see the online supplement for details*).

Histopathology

Unlabeled lungs were fixed via the trachea with 10% buffered formalin at 20 cm H₂O and paraffin-embedded sections were prepared, sectioned, stained with hematoxylin and eosin, and evaluated by pathologists (P.G.I. and S.S.C.) using a BH2 Olympus microscope (Olympus, Tokyo, Japan).

Statistics

Statistical analysis was by *t* test or Mann-Whitney nonparametric analysis as appropriate, using Prism 4.0 software (GraphPad, Inc., San Diego, CA). Survival was calculated by Kaplan-Meier followed by log-rank analysis. Differences were considered significant if *P* < 0.05.

RESULTS

Increased Survival after High-Dose Pneumococcal Infection in gp91^{phox}^{-/-} Mice

gp91^{phox}^{-/-} and C57BL/6 mice received a high-dose pulmonary challenge with pneumococci, previously shown to establish pneumonia (3), and were monitored for 10 days. Any mice that survived for 10 days were deemed to have resolution of pneumonia. As shown in Figure 1, mortality was decreased in gp91^{phox}^{-/-} mice compared with C57BL/6 mice. Seventeen of 25 (68.0%) C57BL/6 mice died. In contrast, 9 of 26 (34.6%) gp91^{phox}^{-/-} mice died.

Increased Clearance of Bacteria from the Lungs of gp91^{phox}^{-/-} Mice

To determine whether improved survival of gp91^{phox}^{-/-} mice was associated with altered microbial clearance we measured bacterial numbers in the lung and blood during pneumonia. In keeping with their increased survival, gp91^{phox}^{-/-} mice had increased pulmonary clearance of bacteria, with significantly

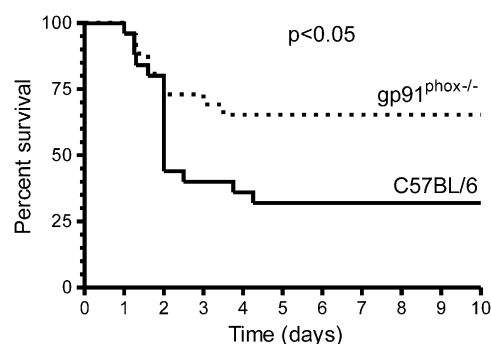


Figure 1. Increased survival in gp91^{phox}^{-/-} mice after pneumococcal infection. Mice were instilled via the trachea with 10⁷ cfu of type 2 pneumococci and monitored for 10 days. There was significantly greater survival of the gp91^{phox}^{-/-} mice (*n* = 26) compared with the wild-type C57BL/6 control mice (*n* = 25); log-rank analysis.

fewer colony-forming units in lung homogenates and in the blood (Figure 2). Because pneumococci can form biofilms, and to exclude the possibility that decreased colony counts in the lung were due to different degrees of biofilm formation between *gp91^{phox}^{-/-}* mice and C57BL/6 mice, we performed Gram stains on lung homogenates diluted in PBS. Although occasional clumps of bacteria were seen there was no overall difference between a series of samples from the two murine strains (data not shown), suggesting the 1-log difference in colony-forming units was not likely due to differential rates of bacterial biofilm formation (17). In these high-dose infection models alveolar macrophages are unable to control infection (3) and the differences observed were likely due to differences in recruited innate immune cells. To determine whether the increased clearance of bacteria was specific to lung infection we also measured colony counts in the blood in a systemic infection model after intraperitoneal instillation of bacteria. As shown in Figure E1 (*see the online supplement*), *gp91^{phox}^{-/-}* mice infected by this route also had significantly lower bacterial colony counts.

Increased Number of Neutrophils in *gp91^{phox}^{-/-}* Mice

With evidence of early enhancement of bacterial clearance in a model that requires recruited inflammatory cells, we next examined the contribution of quantitative differences in neutrophil responses to high-dose pneumococcal challenge in *gp91^{phox}^{-/-}* mice. Decreased phagocyte NADPH oxidase function is associated with enhanced neutrophil recruitment after induction of inflammation (14), which could account for the greater bacterial clearance. Histopathologic sections revealed greater numbers of neutrophils in the lungs of *gp91^{phox}^{-/-}* mice (Figures 3A–3D) and this was confirmed by semiquantitative scoring of tissue sections (Figures 3E and 3F) and by assessment of myeloperoxidase content of lung homogenates (Figure 3G). Infection with pneumococci caused recruitment of neutrophils into the airways of both strains of mice, with significantly greater numbers of neutrophils being seen in the BAL of *gp91^{phox}^{-/-}* mice than in the BAL of C57BL/6 mice at both 24 hours (Figures 4A and 4B) and 48 hours (Figures 4C and 4D) postinfection. Similar findings were demonstrated with type 1 pneumococci, which also produced greater numbers of neutrophils in the lungs of *gp91^{phox}^{-/-}* mice (data not shown). Mice mock infected with PBS had few neutrophils in the BAL, with no significant difference between C57BL/6 and *gp91^{phox}^{-/-}* mice (C57BL/6 mice, $1.3 \times 10^3 \pm 0.7 \times 10^3$ [$n = 9$]; *gp91^{phox}^{-/-}* mice, $1.3 \times 10^4 \pm 0.4 \times 10^4$ [$n = 9$]). Although there was a trend toward more

neutrophils in the *gp91^{phox}^{-/-}* mice, the numbers were small. There was also no difference in circulating neutrophils or total white blood cells (data not shown), as previously reported (14), suggesting that the increased numbers of neutrophils in the BAL were a consequence of increased recruitment or decreased apoptosis of recruited cells or a combination of both processes.

Altered Patterns of Pulmonary Expression of Cytokines/Chemokines Associated with Neutrophil Recruitment during Pneumococcal Pneumonia

TNF- α is a critical early cytokine required for neutrophil recruitment during pneumococcal pneumonia (18). Expression was increased 24 and 48 hours after infection, and the level was sustained to a much greater extent at 48 hours in the *gp91^{phox}^{-/-}* mice (Table 1). Critical CXC chemokines associated with increased TNF- α expression and neutrophil chemotaxis during pneumococcal infection of the lung, KC (CXCL1) and MIP-2 (CXCL2) (19), were also increased (Table 1). As previously reported, MIP-2 production peaked before KC production (18). Because monocyte-derived chemokines such as MCP-1 (CCL2) can amplify neutrophil recruitment in the lung, MCP-1 expression was also investigated and enhanced levels of expression were noted, particularly at later time points after infection, in *gp91^{phox}^{-/-}* mice relative to wild-type mice (Table 1). There was also a significant increase in the amount of IL-6, a pleiotropic cytokine, in BAL from the *gp91^{phox}^{-/-}* mice and the level of expression was sustained for 48 hours (Table 1). These observed increases were seen despite reduced numbers of bacteria in the lungs of *gp91^{phox}^{-/-}* mice and were not observed for all cytokines investigated; there was no difference in levels of IL-12 (Table 1) or IL-2, IL-4, IL-5, IL-10, or IFN- γ (data not shown).

Decreased Neutrophil Apoptosis in *gp91^{phox}^{-/-}* Mice

Impaired NADPH oxidase function in activated neutrophils is associated with reduced apoptosis induction (20). Levels of apoptosis were estimated in BAL 24 and 48 hours postinfection. Cytospins from BAL showed a reduction in apoptosis in *gp91^{phox}^{-/-}* mice compared with C57BL/6 mice (Figures 5A–5C). Levels of neutrophil and macrophage apoptosis were also examined by annexin-V/TO-PRO-3 staining and flow cytometry, with the separate cell populations determined by forward/side scatter characteristics and Ly6G staining to identify neutrophils. There was a significant reduction in annexin-V-PE⁺/TO-PRO-3⁺ apoptotic neutrophils (Figures 5D and 5E), with a similar difference when dual annexin-V-PE⁺/TO-PRO-3⁺ cells were studied (data not shown). Similarly, there were reduced levels of apoptosis as assessed by Ly6G⁺/Hoechst⁺ neutrophils, which persisted for at least 48 hours postinfection (*see Figure E2*). Macrophage apoptosis was not significantly different between the two strains of mice postinfection (*see Figure E3*).

Increased Activation of PMNs from *gp91^{phox}^{-/-}* Mice

Neutrophil activation is required for NADPH oxidase assembly and surface expression of CD11b/CD18, required for adhesion of neutrophils to endothelial cells during inflammatory responses, is an established marker of neutrophil activation (21). To see whether any difference in activation was apparent, CD11b and CD18 surface expression was measured on neutrophils from BAL. Neutrophils from *gp91^{phox}^{-/-}* mice showed greater activation than those from C57BL/6 mice, with higher levels of CD11b (Figure 6A) and CD18 surface expression (Figure 6B). In addition, there was lower surface expression of CD62L on neutrophils from *gp91^{phox}^{-/-}* mice, as compared with those from C57BL/6 mice after infection, in keeping with

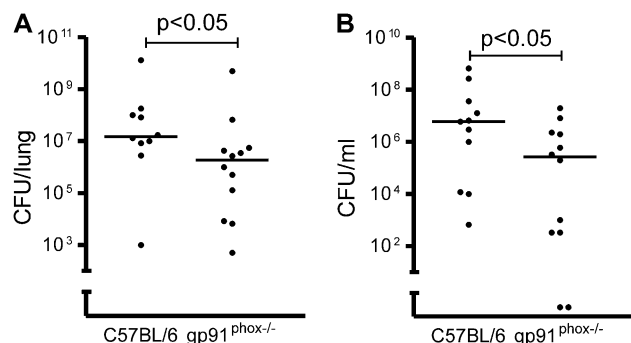


Figure 2. Reduced bacteria in lung and blood from *gp91^{phox}^{-/-}* mice after high-dose pneumococcal infection. Bacteria in (A) lung homogenates and (B) blood from wild-type C57BL/6 control mice and *gp91^{phox}^{-/-}* mice were determined 24 hours after intratracheal instillation of 10^7 cfu of type 2 pneumococci. Means and SEM, Mann-Whitney.

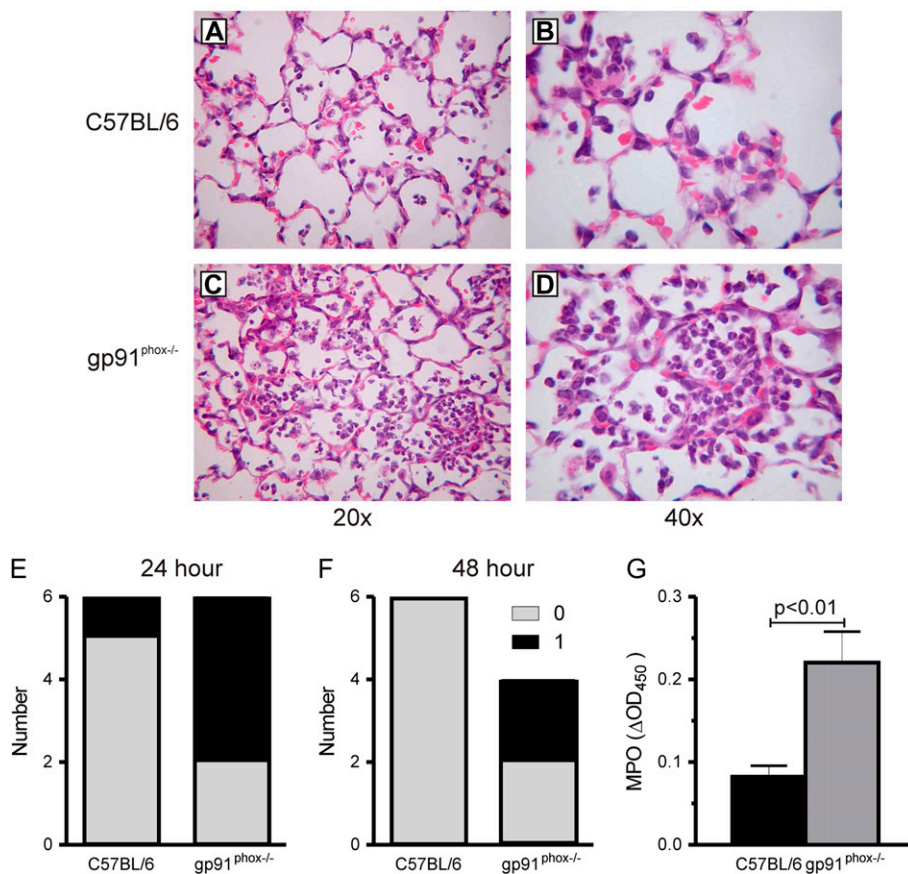


Figure 3. Increased numbers of neutrophils in lung sections from gp91^{phox-/-} mice after infection with pneumococci. Shown are images of lung sections obtained from (A and B) wild-type C57BL/6 control mice and (C and D) gp91^{phox-/-} mice 48 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci. Original magnification: (A and C) ×20; (B and D) ×40. Histology shows changes of patchy lobar consolidation with increased cellularity and increased numbers of neutrophils in the alveolar inflammatory infiltrate of the gp91^{phox-/-} mice. (E and F) Semiquantitative scoring of neutrophilic infiltration in tissue sections (E) 24 hours (n = 6) and (F) 48 hours (gp91^{phox-/-} mice, n = 4; C57BL/6 mice, n = 6) after intratracheal instillation of 10⁷ cfu of type 2 pneumococci, showing numbers of sections with areas of confluent alveolar neutrophilic infiltration in the alveoli (area < 0.5 mm, score 0; area > 0.5 mm, score 1). (G) Myeloperoxidase (MPO) activity in lung homogenates from C57BL/6 and gp91^{phox-/-} mice 24 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci; n = 7 (each group). ΔOD₄₅₀ = change in optical density at 450 nm. Means and SEM, *t* test.

a significant increase in the shedding of CD62L and enhanced activation (Figure 6C). These findings suggested that reduced susceptibility to apoptosis was not associated with decreased activation.

Investigation of Lung Injury in gp91^{phox-/-} Mice

As an increase in neutrophil numbers can be associated with increased vascular permeability and lung injury in models of pulmonary inflammation (6), we performed several assays to investigate these. Despite the greater number of neutrophils in gp91^{phox-/-} mice, there was no significant difference in albumin or IgM levels in BAL between the two strains (Figures 7A and 7B). Similarly, there was no difference in the ratio of wet to dry lung weights (Figure 7C). There was no evidence of significant lung injury in either strain after review of histology sections, although this analysis does not exclude subtle degrees of ultrastructural difference, which would require analysis by a technique such as electron microscopy.

Analysis of Neutrophil Elastase Production in gp91^{phox-/-} Mice

Because some of the consequences of NADPH oxidase activation may be mediated by the resultant activation of granule-associated proteases rather than by ROS directly (7), we examined neutrophil elastase activity in the lungs of both murine strains. Interestingly, as shown in Figure 8A, we observed no decrease in neutrophil elastase activity in the lungs of gp91^{phox-/-} mice. There was less neutrophil elastase in gp91^{phox-/-} mice when values were normalized to cell numbers, but the overall level correlated with the total number of neutrophils (see Figure E4). Although this analysis was of BAL, rather than total lung, it is of relevance, because analysis of neutrophil numbers in BAL provided the same conclusion as analysis of lung homogenates by myeloperoxidase. This suggests that the greater neutrophil numbers compensated for the decreased levels of per-cell production in gp91^{phox-/-} neutrophils. Because pneumococci also produce ROS we wondered whether the generation

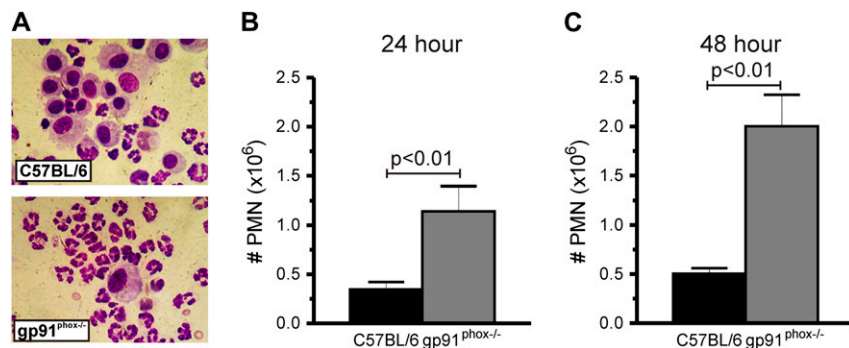


Figure 4. Increased numbers of neutrophils in bronchoalveolar lavage (BAL) from gp91^{phox-/-} mice after infection with pneumococci. (A) Representative appearances of cytopins of BAL from C57BL/6 mice (top) and gp91^{phox-/-} mice (bottom) 24 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci (*Streptococcus pneumoniae*). (B) Absolute numbers of neutrophils (#PMN) in BAL from wild-type C57BL/6 control mice (n = 11) and gp91^{phox-/-} mice (n = 12) from the same experiments as in (A). (C) Absolute numbers of neutrophils in BAL 48 hours after instillation of 10⁷ cfu of type 2 *S. pneumoniae*; n = 5 (each group). Means and SEM, *t* test.

TABLE 1. LEVELS OF CYTOKINES IN BRONCHOALVEOLAR LAVAGE FLUID

	24 Hours			48 Hours		
	C57BL/6	gp91 ^{phox} ^{-/-}	P Value	C57BL/6	gp91 ^{phox} ^{-/-}	P Value
TNF- α	123.0* (65.50–192.0) [†] 5 [‡]	175.0 (123.0–222.0) 7	NS	320.0 (307.0–582.0) 11	733.5 (457.5–957.5) 10	<0.05
KC (CXCL1)	46.52 (38.59–86.35) 10	162.3 (38.15–234.0) 9	NS	40.11 (33.53–169.5) 24	89.82 (41.46–3745) 18	<0.05
MIP-2 (CXCL2)	187.3 (135.3–259.4) 10	424.1 (189.0–604.5) 9	< 0.05	101.8 (55.67–275.9) 24	149.2 (101.3–1469) 18	<0.05
MCP-1 (CCL2)	112.1 (83.69–227.9) 10	158.5 (88.84–421.6) 9	NS	92.94 (70.38–162.1) 24	284.1 (193.4–1537) 18	<0.001
IL-6	526.8 (384.1–755.1) 5	1079 (252.0–1637) 5	NS	168.8 (142.3–182.4) 5	3000 (332.7–3000) 5	<0.05
IL-12 p70	36.52 (32.46–43.27) 10	26.70 (25.48–34.31) 9	NS	33.82 (26.09–44.00) 24	43.60 (33.46–46.55) 18	NS

Definition of abbreviations: MCP-1 = monocyte chemotactic protein-1; MIP-2 = macrophage inflammatory protein-2; NS = not significant; TNF- α = tumor necrosis factor- α .

* Median.

[†] Interquartile range.

[‡] Number of mice.

of bacteria-produced ROS, by inducing changes in ion flux and/or pH, could compensate for the defect in host production. Using the SpxB⁻ mutant, which lacks pyruvate oxidase and

produces less than 2.3% of the H₂O₂ produced by the parental strain (22), we examined whether residual generation of ROS by pneumococci influenced neutrophil elastase release. As

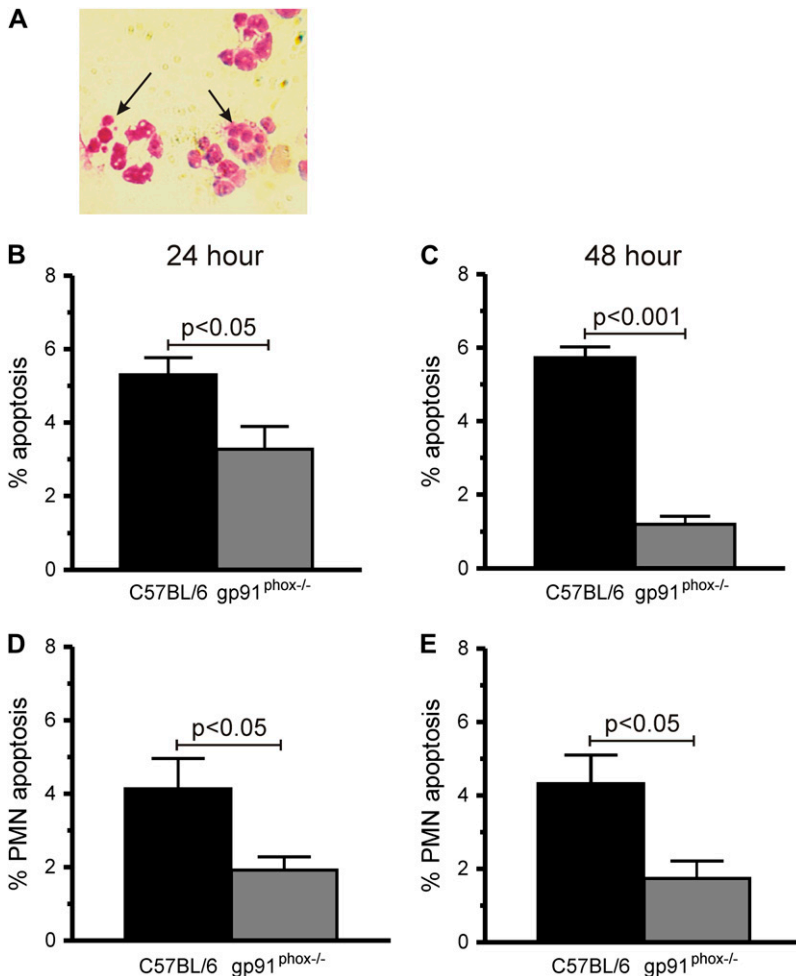


Figure 5. gp91^{phox}^{-/-} mice have reduced numbers of apoptotic cells in the lung after infection with type 2 pneumococci. (A) Representative cytopsin demonstrating apoptotic cells (arrows) of bronchoalveolar lavage (BAL) from C57BL/6 mice 24 hours after instillation of 10⁷ cfu of type 2 pneumococci D39 (*Streptococcus pneumoniae*). (B and C) Percentage of apoptotic events (apoptotic cells and bodies) in cytopsin of BAL from wild-type C57BL/6 control mice (n = 11) and gp91^{phox}^{-/-} mice (n = 12) (B) 24 hours and (C) 48 hours after *S. pneumoniae* instillation; n = 5 (each group). (D and E) Percentage neutrophil (PMN) apoptosis (annexin-V-PE⁺/TO-PRO-3⁻, flow cytometry) in BAL from C57BL/6 and gp91^{phox}^{-/-} mice (D) 24 hours and (E) 48 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci; n = 5 (each group). Means and SEM, *t* test.

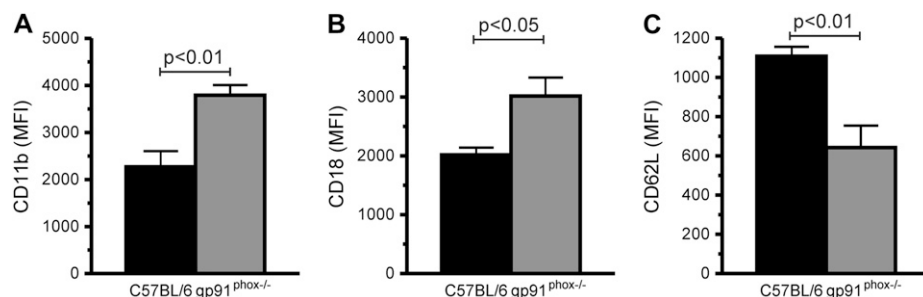


Figure 6. Increase in neutrophil activation in gp91^{phox-/-} mice after infection with pneumococci. Mean fluorescence intensity (MFI) of (A) CD11b, (B) CD18, and (C) CD62L in bronchoalveolar lavage (BAL) from wild-type C57BL/6 control mice and gp91^{phox-/-} mice 24 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci, n = 4 (each group). Means and SEM, t test.

shown in Figure 8A the presence or absence of pyruvate oxidase in the pneumococcus had no impact on the level of neutrophil elastase and the presence or absence of pneumococcal oxidase did not influence total neutrophil numbers (Figure 8B). There was also a trend toward increased clearance of the SpxB⁻ mutant in the lungs of gp91^{phox-/-} mice, although this was less marked than we had observed with the parental strain described above (see Table E1).

DISCUSSION

These results demonstrate that NADPH oxidase-derived ROS, a central component of neutrophil antimicrobial defenses, are not required for pneumococcal killing. Instead, the key role of ROS produced by NADPH oxidase in this infection is to down-regulate the recruitment, activation, and survival of neutrophils. Surprisingly, although gp91^{phox-/-} mice had increased numbers of activated neutrophils in their lungs, the presence of these cells did not result in increased lung injury despite preserved levels of granule-associated proteases, and the overall effect of deficient NADPH oxidase-dependent ROS production was enhanced survival. Our findings suggest that NADPH oxidase-derived ROS are not required for antimicrobial host defense against certain common pathogens. In addition, activated neutrophils do not invariably cause significant lung injury and, in the absence of functional NADPH oxidase, may be well tolerated in the lung. In pneumococcal infection NADPH oxidase activation regulates the inflammatory response but not microbial killing. This is not unique to the lung and the observation of sterile abscesses in chronic granulomatous disease (CGD) suggests these principles also apply to other infections (23).

CGD, a heterogeneous condition characterized by genetic mutations involving subunits of the phagocyte NADPH oxidase complex, classically predisposes to infections with catalase-positive organisms (23). These microorganisms are able to degrade the H₂O₂ produced during their own metabolism. Catalase-negative organisms lack this ability and, in theory, the H₂O₂

produced by catalase-negative organisms can substitute for defective phagocyte production of ROS. Nevertheless, gp91^{phox-/-} mice have been shown also to have defective pulmonary killing of catalase-negative organisms, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, *in vivo* (24–26), and thus our finding of no defect in pneumococcal killing in gp91^{phox-/-} mice was unexpected. In keeping with our findings, infection with the pneumococcus, a catalase-negative organism, is rare in CGD (27). Neutrophils from individuals with CGD have no defect in pneumococcal killing *in vitro* (28, 29) and ROS produced by pneumococci substitutes for host-derived ROS in pneumococcal killing by neutrophils *in vitro* (28). We did not see greater numbers of bacteria in the lung after infection with the SpxB⁻ mutant pneumococcus, which suggests that, in our *in vivo* studies, alternative mechanisms may include non-NADPH oxidase-produced ROS, and antimicrobial molecules, such as proteases and cationic peptides (7, 30, 31).

Genetically modified mice may have compensatory adaptations that lead to unanticipated findings. This can be compounded by the use of control mice that are not heterozygous littermates but may occur even when control mice are littermates. LFA-1^{-/-} mice, although more susceptible to infection in a variety of models, have enhanced clearance of pneumococci after intravenous infection because the gene knockout is associated with greater numbers of neutrophils and greater levels of pneumococcus-specific antibody (32). However, understanding these compensatory changes can be informative and it is possible that other approaches, such as the use of small molecular inhibitors, may also trigger these changes. In addition, the finding of greater neutrophil numbers in response to infection is not unique to our model of CGD, whereas the improved bacterial clearance, to our knowledge, is unique (24, 25).

A major effect of NADPH oxidase-generated ROS in this pneumococcal pneumonia model was to limit neutrophil recruitment, activation, and survival (although the slight difference between baseline numbers of neutrophils, albeit at low levels, means that we cannot completely exclude a small

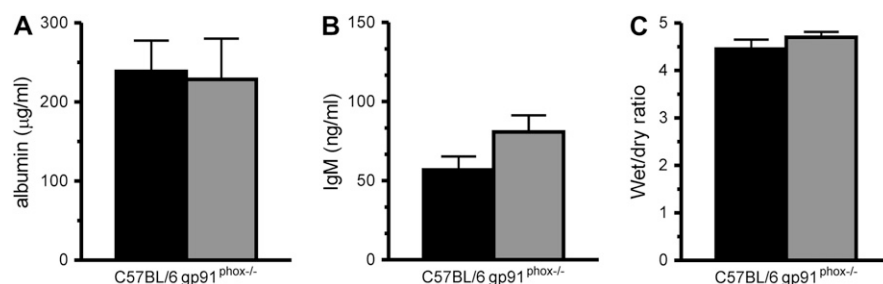


Figure 7. No increase in lung injury in gp91^{phox-/-} mice after high-dose pneumococcal infection. (A) Levels of albumin in bronchoalveolar lavage (BAL) from C57BL/6 (n = 11) and gp91^{phox-/-} (n = 10) mice, 48 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci. (B) Levels of IgM in BAL from C57BL/6 (n = 23) and gp91^{phox-/-} (n = 16) mice 24 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci. (C) Wet-to-dry ratio of lungs from C57BL/6 and gp91^{phox-/-} mice 24 hours after intratracheal instillation of 10⁷ cfu of type 2 pneumococci, n = 6 (each group). Means and SEM, Mann-Whitney.

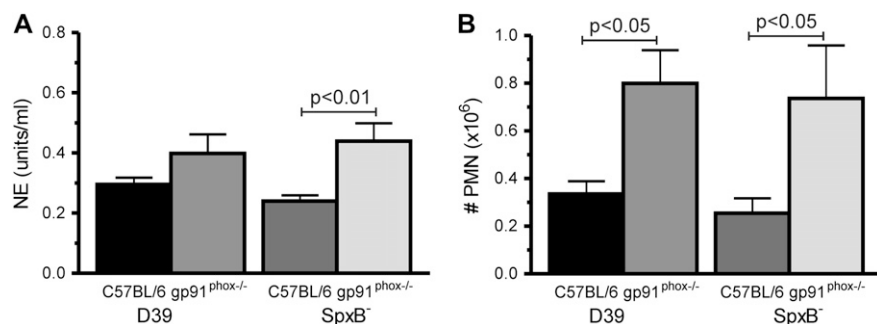


Figure 8. Neutrophil elastase production in gp91^{phox-/-} mice. (A) Levels of neutrophil elastase (NE) in bronchoalveolar lavage (BAL) from C57BL/6 and gp91^{phox-/-} mice 24 hours after instillation of 10⁷ cfu of type 2 pneumococci D39 or pneumococci lacking pyruvate oxidase activity (SpxB⁻; n = 7 (each group)). (B) Absolute numbers of neutrophils in BAL from the same experiments as in (A). Means and SEM, Mann-Whitney.

contribution from baseline differences in neutrophil numbers in the lung). Each of these processes could contribute to clearance, but the current study does not allow us to determine the relative contribution of each in the current model. ROS contribute to cell signaling via nuclear factor- κ B, activator protein (AP)-1, and mitogen-activated protein kinases (33). gp91^{phox-/-} mice had increased production of proinflammatory cytokines and chemokines and these will also have modified neutrophil phenotype indirectly. The sustained production of CXC chemokines is noteworthy because inhibition of ROS signaling by antioxidants has previously been associated with down-regulation of CXC chemokine expression (34), suggesting in our model a shift to ROS-independent chemokine expression. The neutrophil activation status reflects the proinflammatory cytokine environment and the up-regulation of β_2 -integrin, which, in the absence of ROS generation (35), stimulates prosurvival pathways (36). Delayed constitutive and Fas-mediated apoptosis occurs in neutrophils from patients with CGD (37). Decreased levels of apoptosis of neutrophils from patients with CGD are associated with decreased production of antiinflammatory mediators prostaglandin D₂ and transforming growth factor- β (38). Thus, inhibition of apoptosis has indirect effects mediated by downstream cytokines/chemokines.

Activated neutrophils contribute to acute lung injury (6, 39), but increased neutrophils in the gp91^{phox-/-} mice did not cause gross lung injury. However, they were associated with increased clearance of bacteria and experiments using a model of peritoneal infection also confirmed enhanced clearance of bacteria by gp91^{phox-/-} mice, demonstrating that the increased clearance is not unique to the pulmonary route of infection. Neutrophil depletion (40) and inhibition of Fas-dependent neutrophil recruitment (41) have improved specific microbiological outcomes in mice with pneumococcal pneumonia, but the parameters affected have varied and results have been dose specific. We extend these findings by making the observation that inhibition of NADPH oxidase negates some of the harmful effects of neutrophils in pneumonia models. Decreasing neutrophil numbers protect against the negative effects of NADPH oxidase activation but potentially at the expense of limiting NADPH oxidase-independent neutrophil killing of pneumococci in the lungs. This may explain why gp91^{phox-/-} mice had greater clearance of bacteria in the lungs whereas neutrophil depletion preferentially decreased the level of bacteremia (40). Our findings suggest a novel approach to pneumococcal pneumonia may be the selective manipulation of NADPH oxidase activation.

Surprisingly, gp91^{phox-/-} mice had no evidence of decreased neutrophil elastase because increased numbers of neutrophils compensated for the reduction of neutrophil elastase released per cell. Nor was the neutrophil elastase release in gp91^{phox-/-} mice the consequence of bacterially produced ROS. Potential explanations for the lack of granule-associated protease-induced acute lung injury include evidence that ROS inactivate

protease inhibitors such as α_1 -antiprotease (42). In keeping with this, our preliminary data have shown evidence of increased levels of secretory leukoprotease inhibitor, an inhibitor of neutrophil-expressed serine proteases, in gp91^{phox-/-} mice (see Figure E5). Neutrophil elastase can modify the inflammatory response by enhancing IL-8 production and inducing shedding of surface expressed receptors (43, 44).

Previous studies using antioxidants in pneumococcal infections of the lung have shown mixed results, although increased bacterial clearance along with increased neutrophil numbers has been demonstrated in a mouse model after vitamin C treatment (45). However, antioxidants do not selectively inhibit NADPH oxidase activation. Prior models enforce the concept that manipulation of the NADPH oxidase complex does not produce the same phenotype as antioxidant treatment after infection (46). A future therapeutic approach would be to specifically target the neutrophil NADPH oxidase complex, potentially by targeting regulatory small GTPases (47). Selective inhibition of Rac2 would be a suitable target because it is required to activate the NADPH oxidase complex and loss-of-function mutations in this molecule replicate the CGD phenotype (48).

Because our findings relate specifically to pneumococcal infection, rapid microbiological diagnostics would be essential to identify patients who present with severe community-acquired pneumonia caused by pneumococci. Only a subset of individuals with disease will be expected to have a poor outcome, and thus biomarkers of the degree of ROS generation via NADPH oxidase will be required (49). Suitable patients would then be selected to receive adjunctive therapy, to manipulate their NADPH oxidase activation, with the aim of improving disease outcomes. Further studies both in model systems and in appropriate populations of patients with pneumonia will be required to determine the utility of such an approach.

Conflict of Interest Statement: H.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.E.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.S.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.J.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.S.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.G.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.G.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.K.B.W. has received a research grant from GlaxoSmithKline, relating to a multicenter asthma genetics study. She has received support from Boehringer Ingelheim for conference attendance and lecture fees from AstraZeneca. D.H.D. has received support from GlaxoSmithKline, Gilead, Boehringer Ingelheim, Abbott, and Roche for conference attendance and has received lecture fees from GlaxoSmithKline.

Acknowledgment: The authors thank Sue Newton and Jennifer Gammon for help with the cytometric bead array assay.

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